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Prostate Cancer (PCa) accounts for nearly 30% of all newly diagnosed cancers among American men. Epidemiologic studies suggest that dietary factors may be important in the etiology of PCa. The objectives of our research is to determine how nutritional compounds genistein, betasitosterol, and omega-6 fatty acids function as modulators of PCa. In the first year, we are developing the technical tools with which to investigate gene expression patterns that are altered by the 3 dietary compounds. We have upgraded and optimized our microarrayer to allow for more efficient printing. The new microarrayer setup will aid in preparing a custom microarray containing approximately 400 unique cDNAs that are relevant in pathways of apoptosis and androgen receptor among others. In addition, we optimized our protocol for multicolor spectral transcript analysis to accommodate up to 8 colors so as to maximize the number of different RNA species that can be detected. To test the improved tools, we have initiated in vitro experiments using PCa cell lines and the three dietary compounds. In addition, the fellow has learned xenograft techniques in the co-mentor's laboratory as well as biostatistical analyses that will be applied towards the in vivo studies for the second year of fellowship.

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Introduction

Prostate Cancer (PCa) accounts for nearly 30% of all newly diagnosed cancers among American men. Epidemiologic studies suggest that dietary factors may be important in the etiology of this disease. The objective of our research is to determine how nutritional compounds genistein, betasitosterol (SIT), and omega-6 fatty acids (FA) function as modulators of PCa. These three compounds belong to three distinct classes of dietary components, specifically isoflavonoid (genistein), phytosterol (SIT), and FA (omega-6 FA). Each class of compound could potentially modulate PCa in different ways. Previous investigations have indicated that all three compounds may affect PCa in a dose-dependent fashion. We will determine differences in gene expression profiles at a range of doses on *in vitro* models. We will then test two doses on *in vivo* models to account for the complexity of tumor microenvironments.

In the first year, we are developing the technical tools with which to investigate gene expression patterns that are modulated by the 3 dietary compounds. We have upgraded and optimized our microarrayer to allow for more efficient printing. The new microarrayer setup will aid in our preparation of a custom microarray containing approximately 400 unique cDNAs that are relevant in pathways of apoptosis and androgen receptor among others. We have also improved our microarray protocol to be able to detect gene expression patterns of less than 100 cells. In addition, we are optimizing our protocol for multicolor spectral transcript analysis to accommodate up to 8 colors so as to maximize the number of different RNA species that can be detected. To test the improved technical tools, we have initiated *in vitro* experiments using PCa cell lines and the three dietary compounds. In preparation for the second year of fellowship, the fellow has learned xenograft techniques in the co-mentor's laboratory as well as biostatistical analyses that will be applied towards the *in vivo* studies.

Body

Here, we report our progress as it relates to the approved Statement of Work:

Task 1: To delineate the gene expression patterns of PCa in vitro of three nutritional compounds. (Months 1-15)

1.1 Obtain cDNAs for microarrays (Months 1-4)

We initially chose 200 genes (involved in pathways of, i.e., androgen receptor, apoptosis, cell cycle control, etc.) as features for our custom microarray. However, after re-evaluating the pathways, we decided to expand our panel of genes to 400 so as to include a more comprehensive set of genes that are important in the molecular pathways to PCa (Gonzalgo and Isaacs, 2003). From our in-house resources, we were able to obtain only 200 usable unique cDNA clones. The insert sizes were verified by restriction enzyme digestion and/or PCR. Of these 200 clones, we are sequence-verifying 20 (or 10%) of the clones at the UC Berkeley Biochemical Core Facility.

Due to the lack of in-house resources to obtain the remaining 200 genes, we are experiencing a delay in completing our cDNA set for microarray printing. Currently, we are purchasing the remaining unique cDNA clones through ATCC. The majority of these clones is from the Mammalian Gene Collection or has been sequence verified by ATCC. We will verify insert sizes of each clone ordered by restriction enzyme digestion and/or PCR. We will also be sequence-verifying 10% of the unsequenced clones.

1.2 Make arrays and optimize protocols (Months 3-6)

We have recently upgraded our microarrayer with improved hardware (new style of printing pins and print-head; Figure 1). With this new setup, the fellow has extensively tested the printing system within the last few months to verify that the upgrades are compatible with our older system and can generate a more uniform array (Figure 2). We have also increased the number of pins from 1 to 4 in anticipation of printing larger custom arrays.

Figure 1: Modifications made to the DNA microarrayer. The picture shows the Telechem print head installed on our arrayer just prior to printing a set of genomic DNA micro-arrays. The insert shows an enlarged view of the print head with one Telechem pin installed.

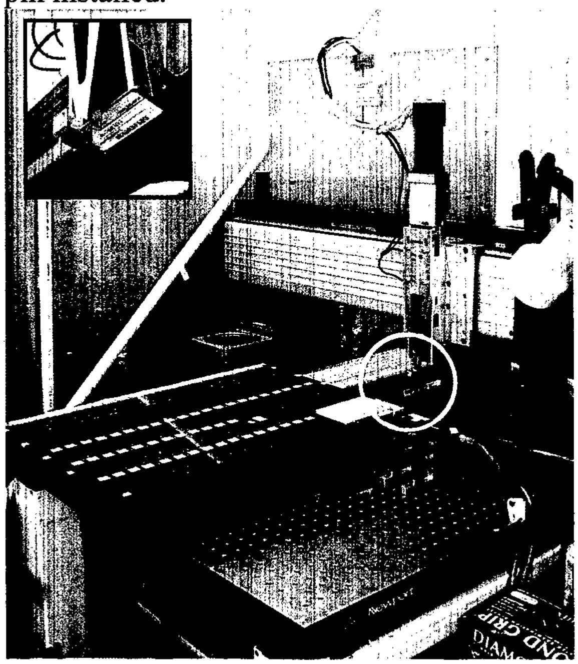
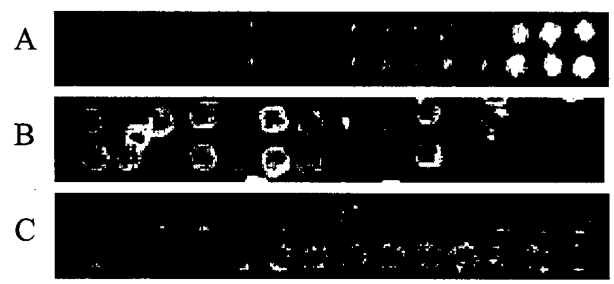


Figure 2: Technical improvements of the upgraded microarrayer. A) Arrays printed with the old (DiTech) pins; B) arrays printed with a Telechem pin on the old holder; C) arrays printed with the same pin as in B installed on the Telechem print head.



1.3 Grow cells under test conditions, isolate RNA, RT-PCR, label probes (Months 3-12)

For this task, we have chosen to use a well-known cell line, LNCaP, as well as three other PCa cell lines (BPH1, BPH1-CAFTD03, and BPH-CAFTD04) generated by the co-mentor. We are currently culturing the cell lines so that all experiments on all cell lines can be performed simultaneously. The range of concentrations of each dietary compound of interest has been determined by a review of current literature on each compound. To parallel the microarray experiments, we will be testing for proliferation (by BrdU incorporation and detection) as well as apoptosis (as detected by caspase assays and/or TUNEL assays).

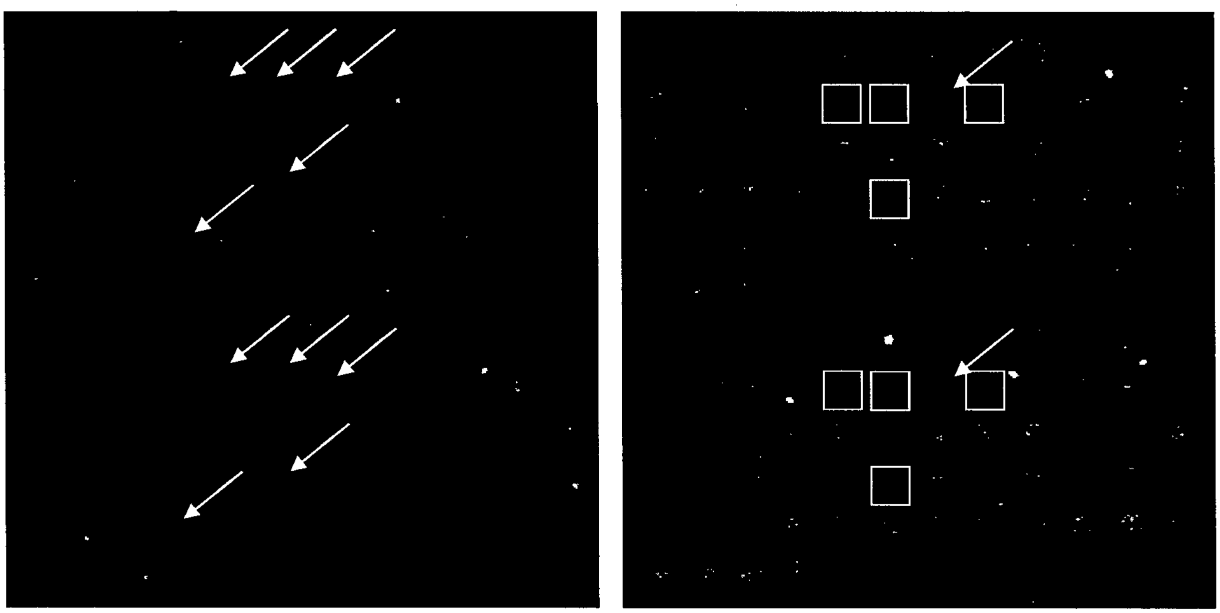
1.4 Perform microarray experiments, analyze data with standard statistical programs (Months 6-14) and Perform multicolor spectral transcript analysis and analyze data with standard statistical programs (Months 10-14)

Due to the delay in obtaining the full 400 cDNA set as well as to the recent modifications of the microarrayer, we have not yet been able to print the custom array. However, the fellow has

used this time to improve upon our microarray protocols and demonstrated the feasibility of microarray analysis using less than 100 dissected breast and prostate cells for microarray analysis after PCR amplification (Figure 3). Briefly, 100 to 1000 cells were dissected from normal and cancer clinical specimens. RNAs from the cells were isolated and cDNAs were generated from the RNAs. cDNA equivalent to 0.3 to 100 cells were used for a 2-step PCR amplification scheme using tyrosine kinase-specific primers. The PCR products were then made into probes by random priming (Invitrogen) to incorporate fluorescent nucleotides. The probes were applied to custom arrays that contained tyrosine kinase genes to determine expression patterns of the targeted genes.

These improved protocols will be essential for using small number of cells that may result from both the *in vitro* and *in vivo* experiments.

Figure 3: Microarray analysis of microdissected breast (A; from 10 cell equivalent PCR products) and prostate (B; from 3 cell equivalent PCR products) cancer specimens. Arrows indicate example RNA species that are alternatively expressed between the test/tumor **(red)** and the control/normal **(green)**. Comparison between A and B panels show some RNA species that are not expressed by prostate cells (squares in panel B) as opposed to breast cells.

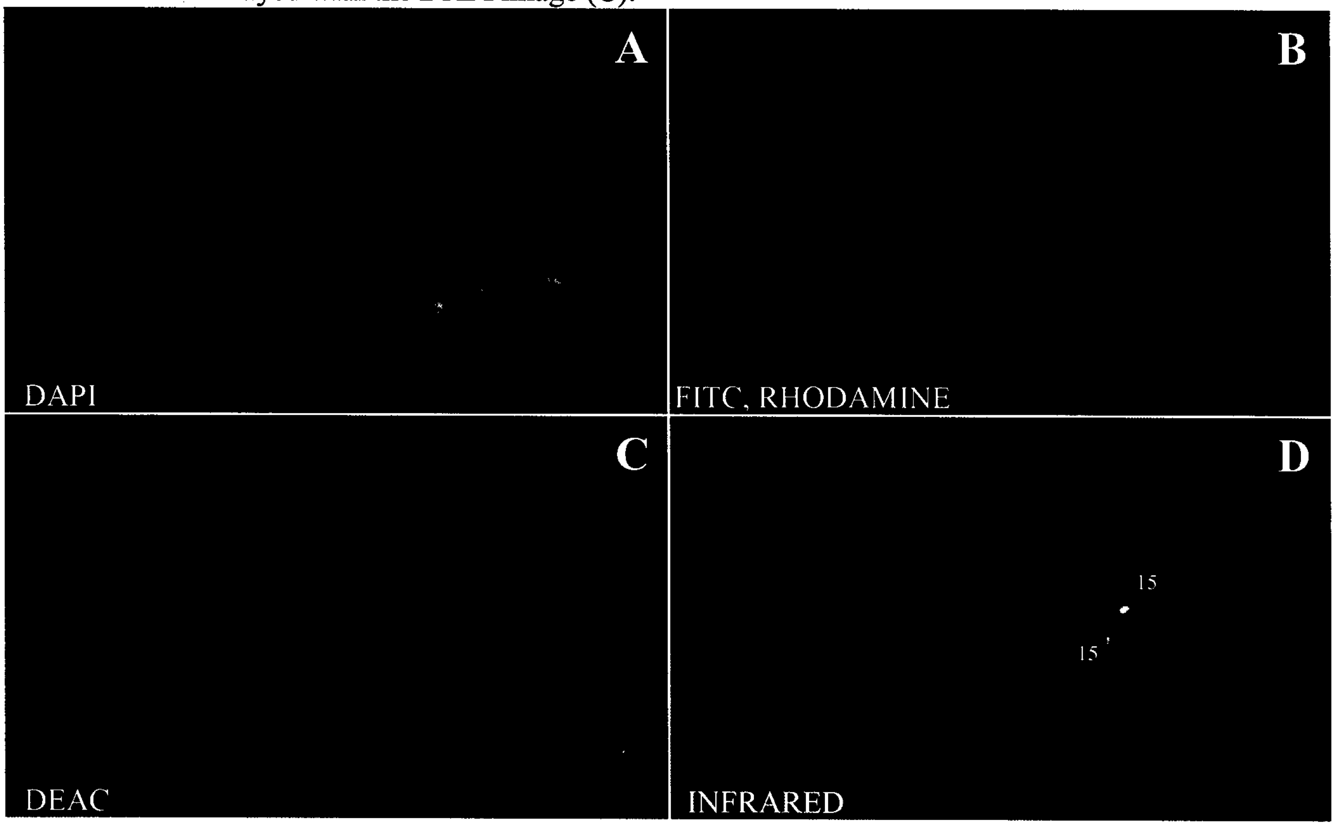


A **B**

In preparation for data analysis, the fellow has taken the "Statistics for Bioinformatics" course at UC Berkeley in the Spring 2003 semester. This course includes lectures on how statistical analysis is applied towards microarray data analysis.

In addition to improvements made to the microarray protocols, we have been optimizing our protocol for multicolor spectral analysis to increase the number of colors detected from 5 to 8 (Figure 4). This modification will allow us to maximize the number of RNA species that can be analyzed by multicolor spectral transcript analysis to parallel the microarray experiments.

Figure 4: Development of a set comprised of 8 uniquely labeled DNA probes. The set of 8 chromosome-specific probes as seen using a filter-based microscope. The Chroma Technology Quad filter set allows excitation and fluorescence imaging of DAPI (A), FITC and Rhodamine (B), and Cy5/Cy5.5 (D). Cy5 and Cy5.5 fluorescence signals were recorded separately and combined with DAPI signals in figure D. The DEAC-labeled probes for chromosome 18 (C) were recorded with a separate filter set matching the DEAC/Spectrum Aqua excitation and emission and overlayed with the DAPI image (C).



Task 2: To determine if and how three select dietary components modulate the growth and gene expression of clinical PCa specimens in vivo. (Months 9-24)

2.1 Pilot study to investigate timing of nutrient supplementation in SCID mice (Months 9-12)

In preparation for *in vivo* experiments, the fellow has learned the surgical xenograft technique on SCID mice at the co-mentor's laboratory using various cell lines. We are preparing custom purified diets for the control and test studies. Once all mouse diets are ready for use, we will implant cell lines used in Task 1.3 for this initial pilot study to determine if there are any differences in gene expression patterns due to differences in diets in an *in vivo* setting. The xenografts will be allowed to grow for 21 days. At day 21, tumors will be harvested and frozen for subsequent tasks to be performed in the second year of fellowship.

2.2 Analyze tumors from pilot study (Months 12-16)

2.3 *Grow 6 tumors in 21 SCID mice (2 tumors per mice) that ingest one of 7 test diets, harvest tissues, prepare tissue sections and RNAs, RT-PCR, and make probes. Test diets include control and each compound at one of 2 concentrations (Months 15-16)*

2.4 *Perform microarray experiments, analyze data with standard statistical programs (Months 17-24)*

2.5 *Perform multicolor spectral transcript analysis and analyze data with standard statistical programs (Months 20-24)*

2.6 *Write final reports and prepare data for publication (Months 22-24)*

Key Research Accomplishments

- Prepared 200 out of 400 unique cDNA clones necessary for printing a custom microarray.
- Upgraded and optimized microarray printing setup and protocols.
- Developed and modified microarray protocols for detecting gene expression in less than 100 cells.
- Optimized a Spectral Imaging-based system to detect up to 8 colors for maximizing the number of RNA species detectable during multicolor spectral transcript analysis.
- Training:
  - Attended biostatistics course in preparation of microarray analysis.
  - Learned surgical techniques for *in vivo* xenograft experiments (2^{nd} year of fellowship).

Reportable Outcomes

- None.

Conclusion

Due to our setback in obtaining the full 400 cDNA set necessary for our custom microarray, we have used the first year of this fellowship for training and optimization of protocols necessary for this project. We have spent a considerable amount of time to improve the tools with which to detect gene expression in our *in vitro* and *in vivo* prostate cancer model systems, as was defined in the statement of work. The optimization of our protocols enables us to be able to print our custom microarray as well as maximize the number of transcripts seen by multicolor spectral transcript analysis. Ongoing *in vitro* studies will provide the first set of samples to test our optimized technologies. In addition, the fellow has been trained in surgical procedures that are essential for *in vivo* experiments as well as statistical analysis that are necessary for completion of the fellowship.

References

Gonzalgo, ML and Isaacs, WB. Molecular Pathways to Prostate Cancer. J Urol. 170(6, Part 1 of 2): 2444-2452. 2003.